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CALYCOSIN 7-*O*-*β*-D-GLUCOPYRANOSIDE, AN ANTI-HIV AGENT FROM THE ROOTS OF *Astragalus membranaceus* VAR. *mongholicus*

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The roots of *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao (Leguminosae) have long been used as traditional Chinese medicine to cure chronic diarrhea, prolapse of the rectum, hematochezia and abnormal uterine bleeding, edema, anemia, albuminuria in chronic nephritis, and diabetes in China [1]. Calycosin 7-*O*-*β*-D-glucopyranoside (**1**) isolated from this medicine possesses multiple biological and pharmacological activities such as an alleviating effect on osteoarthritis [2], a protective effect on rat hepatocytes [3], an inhibitory effect on COX-2 activity [4], and antimicrobial and superoxide anion scavenging activities [5]. Additionally, this compound can also efficaciously increase the fluidity of brain cell membrane in ischemia-reperfusion rats [6, 7].

Natural products provide a large reservoir for screening of anti-HIV-1 agents because of their novel structure, anti-viral properties and structural diversity, such as *β*-galactose-specific lectin [8], propolis [9], betulinic acid [10], a series of 4-phenylcoumarins [11], xanthohumol [12], etc. Unfortunately, the anti-HIV-1 activities of **1** have not been reported. In the present research, cytotoxicity assay, syncytium reduction assay on C8166 cells, and the inhibition assay of HIV-1 induced cytopathogenicity on MT-4 cells for calycosin 7-*O*-*β*-D-glucopyranoside were carried out for the first time.

The phytochemical investigation on *A. membranaceus* led to the isolation and characterization of calycosin 7-*O*-*β*-D-glucopyranoside. Colorless needles (methanol); mp 221~222° C; ESIMS (positive mode) *m/z*: 447 $[M + H]^+$.

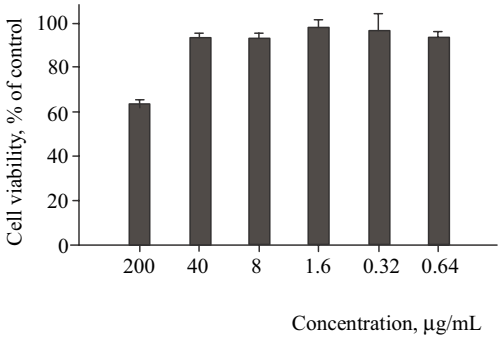

Fig. 1

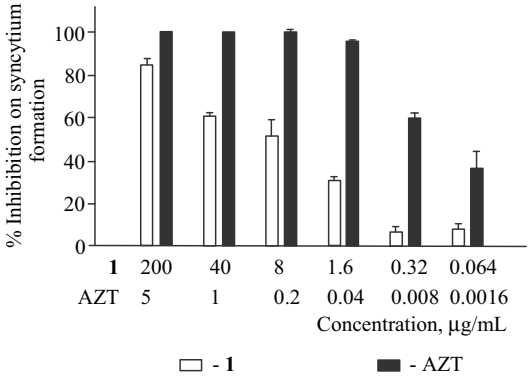

Fig. 2

Fig. 1. Cytotoxicity of calycosin 7-*O*-*β*-D-glucopyranoside on C8166 cells. The cell viability was measured by MTT assay. Data are expressed as means ±SD of triplicate measurements.

Fig. 2. Cytopathic effect inhibition of calycosin 7-*O*-*β*-D-glucopyranoside on C8166 cells induced by HIV-1_B. Data are expressed as means ±SD of triplicate measurements.

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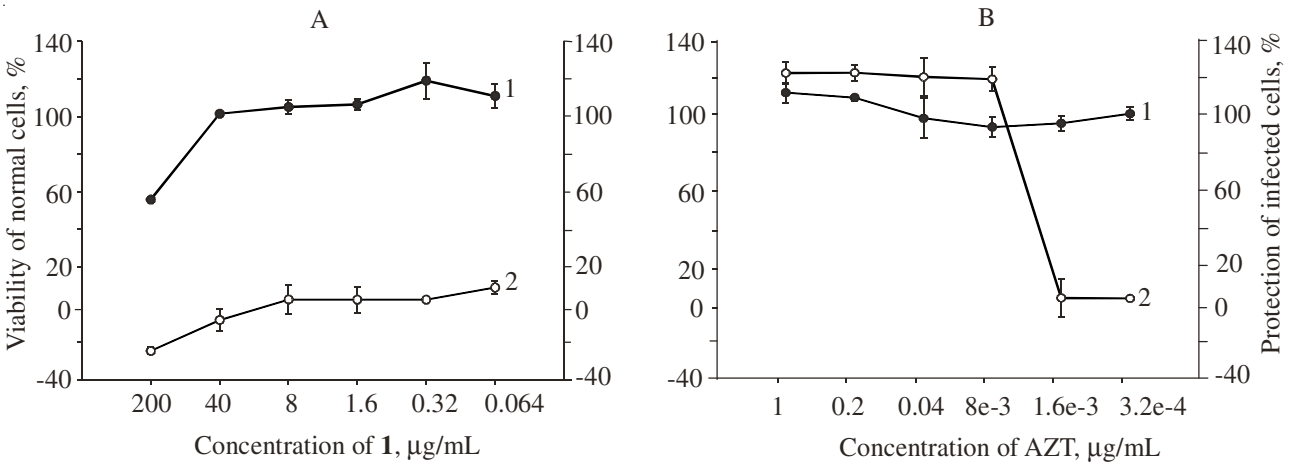

Fig. 3. Protection of calycosin 7-*O*-*β*-D-glucopyranoside (A) and AZT (B) on HIV-1 induced MT-4 cell lytic effects. Data are expressed as mean ±SD of triplicate measurements.

The effect of **1** on the viability of C8166 cells is shown in Fig. 1. This compound exhibited low cytotoxicity on C8166 cells and its CC_{50} value was above 200 μg/mL (448.4 μM).

The virus-induced cytopathic effect was quantified by syncytium formation. The inhibitory activity of **1** on HIV-1 induced syncytium formation (cytopathic effects) in a dose-dependent manner is shown in Fig. 2. The EC_{50} of **1** on inhibiting the cytopathic effects was 7.02 μg/mL (15.74 μM). So the TI (therapeutic index) of this compound against HIV-1 was above 28.49.

The activity of calycosin 7-*O*-*β*-D-glucopyranoside (**1**) and AZT in protecting MT-4 cells from cytopathogenicity induced by $HIV\text{-}1_{IIIB}$ is shown in Fig. 3. Unfortunately, this compound possessed no activity in this aspect. At low concentrations of 0.064 μg/mL (0.14 μM), **1** showed very weak ability, with a protective percentage of 8.95 ± 0.93 %. The protective ability did not increase with concentration but became much lower, probably because of its cytotoxicity effect on the cell lines.

General speaking, any compound with the ability to kill cells will inhibit viral replication, but most of these compounds cannot be qualified as antiviral agents because of their simple cellular massacre. An ideal antiviral agent must be able to kill the infected cells while sparing the normal ones. With the high TI value, calycosin 7-*O*-*β*-D-glucoside is anticipated to be a promising anti-HIV agent.

There are large numbers of natural compounds belonging to the flavonoid family exhibiting anti-HIV-1 effect *in vitro* and often with more than one mode of action. They can interact at different steps in the life cycle of HIV-1, including viral entry [13,14], integrase [15, 16], and Vpr [17]. Both the TI value and the considerably high content (above 0.03%) of **1** in *A. mongholicus* Bge. var. *mongholicus* (Bge.) Hsiao (e.g., grown in Gansu province, China. [18]) make it very valuable to further study the mechanism of anti-HIV activities of this compound, which will be the subject of our future work.

Plant Material. The roots of *A. membranaceus* (Fisch) Bge. var. *mongholicus* (Bge.) Hsiao were purchased from Hunyuan County, Shanxi Province, P. R. China, in January 2002 and identified by Professor C.G. Huang (Shanghai Institute of Materia Medica, Chinese Academy of Sciences). A voucher specimen (ZM-2002018) was deposited at the Herbarium of Shanghai Institute of Materia Medica, Chinese Academy of Sciences (CAS).

Isolation of Calycosin 7-*O*-*β*-D-glucoside (1). Raw roots of *A. membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao (7.0 kg) were extracted with 95% ethanol (25 L × 3, 3 h each time) under reflux. The combined extract was concentrated under reduced pressure to give 950 g residue, which was suspended in H_2O (5.0 L) and fractionated with petroleum ether (3.5 L × 4), EtOAc (3.5 L × 4) and n-butanol (3.5 L × 4) to give corresponding fractions A (1 g), B (24 g), and C (120 g). Fraction B was divided into five subfraction B1–B5 by silica gel column (400 g, 200–300 mesh, 50 × 420 mm), using $CHCl_3$-CH_3OH (100:1, 50:1, 10:1, 5:1, 0:1, each 2500 mL) as eluting solvents. Then, subfraction B4 (1.5 g) was separated over a Sephadex LH-20 column (50 g, 30 × 260 mm) with methanol (800–1200 mL) to give calycosin 7-*O*-*β*-D-glucopyranoside (100 mg): colorless needles (CH_3OH); mp 221–222°C; $[\alpha]_D^{20}$ –40.2° (*c* 0.483, DMSO), R_f 0.50 ($CHCl_3$–CH_3OH–H_2O, 13:7:2); ESIMS (positive mode) *m/z*: 447 $[M + H]^+$; IR (ν_{max}, cm^{-1}): 3394, 2908, 1625, 1510, 1444, 1267, 1203, 1080, 1008, 852, 651; UV (CH_3OH, λ_{max}, nm, log ε): 286 (4.17), 261 (4.38), 220 (4.46), 207 (4.42). D-Glucose was obtained by hydrolyzing the compound in 2 mol/L HCl (aq.) – 95% EtOH and identified by TLC (S_1: R_f 0.30) and PTLC (S_2: R_f 0.28; S3: R_f 0.18; S4: R_f 0.24) with

authentic sample and optical rotation $[\alpha]_D^{20}$ +52.9° (*c* 0.122, H_2O)). Identification of this compound **1** was made by analysis of its' ^1H and ^{13}C NMR spectra in comparison with those of published data [19].

Cell Lines and Virus. Human T-cell lines (C8166, H9) and chronically infected H9/HIV-1_{IIIB} were kindly donated by Medical Research council (MRC), AIDS Reagent Project, UK. All the cell lines and virus were maintained at 37°C with 5% CO_2 in RPMI-1640 medium supplemented with 10% (V/V) heat-inactivated newborn calf serum (Gibco). HIV-1_{IIIB} was prepared from the supernatants of H9/HIV-1_{IIIB} cells. The 50% HIV-1 tissue culture infectious dose ($TCID_{50}$) in C8166 cells was determined and calculated by the Reed and Muench method. Virus stocks were stored in small aliquots at –70 °C. The titer of virus stock was 3.4×10^6 $TCID_{50}$/mL.

Cytotoxicity Assay. The cytotoxicity of the compounds on C8166 cells was determined by MTT colorimetric assay as described previously [20]. Briefly, 100 μL/well (4×10^5/mL) C8166 cell suspension was seeded on a microtiter plate. A 100 μL/well of various concentrations of the compound was added and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 72 h. Discarding 100 μL of the supernatant, MTT reagent was added and incubated for 4 h, and then 100 μL of 50% DMF–10% SDS was added. After the formazan was dissolved completely, the absorbance at 595 nm/630 nm ($A_{595/630}$) was read on an ELISA reader (El×800, Bio-Tek). The cytotoxic concentration that caused the reduction of viable cells by 50% (CC_{50}) was calculated from the dose response curve.

Syncytium Reduction Assay. In the presence of 100 μL of various concentrations of the compound, C8166 cells (4×10^5/mL) were infected with HIV-1_{IIIB} at a multiplicity of infection (M.O.I) of 0.06. The final volume per well was 200 μL. AZT was used for drug control. After 3 days of culture, the cytopathic effect (CPE) was measured by counting the number of syncytia (multinucleated giant cell) in each well under an inverted microscope. The percentage inhibition of syncytial cell formation was estimated from the percentage of syncytial cell number in treated culture to that in infected control culture, and the 50% effective concentration (EC_{50}) was calculated [21].

Protection for HIV-1 Induced Lytic Effects. The activity of the compound against acute HIV-1 infection was based on the inhibition of HIV-1 induced cytopathogenicity in MT-4 cells as described previously [22]. Uninfected or HIV-1_{IIIB} -infected (MOI = 0.1) MT-4 cells (3×10^5 cells/mL) were seeded in 96-well flat-bottomed microtiter culture plates with 100 μL of different concentrations of the compound. AZT was used as the control drug. After 7 days incubation at 37°C, the viability of both HIV-1 and mock-infected cells was assessed by MTT method.

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